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Dixie J. Goss
University of Nebraska - Lincoln

Lawrence J. Parkhurst
University of Nebraska - Lincoln, lparkhurst1@unl.edu

Arkesh M. Mehta
University of Mississippi Medical Center, Jackson, MS

Albert J. Wahba
University of Mississippi Medical Center, Jackson, MS

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The binding of ribosomal protein S1 to S1-depleted 30S and 70S ribosomes. A fluorescence anisotropy study of the effects of Mg^{2+}

Dixie J.Goss and Lawrence J.Parkhurst
Department of Chemistry, University of Nebraska, Lincoln, NE 68588, and

Arkesh M.Mehta and Albert J.Wahba
Department of Biochemistry, University of Mississippi Medical Center, Jackson, MS 39216, USA

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ABSTRACT
We have determined the equilibrium constants for the binding of AEDANS-labelled S1 to S1-depleted 30S and 70S ribosomes. For "tight" ribosomes, the association of S1 increases with the sixth power of Mg^{2+} concentration, but for 30S subunits and "loose" ribosomes, there is virtually no dependence of the association on Mg^{2+} over the same concentration range, 2-10 mM in Mg^{2+}. The binding of S1 to 70S ribosomes at 10 mM Mg^{2+} is stabilized by 2 kcal/mol compared to the binding to 30S subunits. When intact S1 binds to tight ribosomes, the fluorescence anisotropy is that for virtually complete rotational immobilization. The anisotropies vary considerably with the preparation and treatment of both S1 and ribosomes and these variations are detailed here. The results suggest the linkage of Mg^{2+}-dependent conformational changes in the intact ribosomes, perhaps including rRNA, and the binding of S1.

INTRODUCTION

Of the various ribosomal proteins, several of the properties of S1 are noteworthy or unusual. Under some conditions S1 is relatively weakly bound to the 30S subunit and may be exchanged with S1 in solution.[1] The protein may therefore interact with a number of other polypeptide chain initiation components before they bind to the ribosome. S1 is required for initiation of protein synthesis with natural mRNA and stimulates amino acid incorporation when synthetic mRNA is used.[2,3] S1 has been shown to unwind helical segments of RNA,[4-6] but the significance of this with regard to S1 function is not clearly understood.

S1 is also one of the subunits of the phage-induced enzyme Qβ replicase.[7] S1 is the largest ribosomal protein. Its mw, determined from the amino acid sequence, is 61,159.[8] The protein is elongated with about a 10:1 axial ratio.[1] S1 has been

cross-linked to proteins S2, S10 and S18 on the 30S ribosomal subunit.[9,10] Immuno-electron microscopy has localized S1 on the platform-like structure of the 30S subunit.[11,12]

A detailed picture of the role of S1 in protein synthesis is lacking. We have undertaken fluorescence polarization studies in order to compare the binding of S1 to 30S and 70S ribosomes and determine the effects of (Mg^{2+}) on the S1 binding. Previous studies have used ultracentrifugation to estimate these parameters.[1,13] Recently, Chu et al.[14] have used fluorescence polarization to measure S1 binding to ribosomes depleted of S1 by dialysis against low salt buffers. We report here equilibrium constants for S1 binding to S1-depleted 30S subunits and various types of S1-depleted 70S ribosomes as a function of (Mg^{2+}). Studies were made with fluorescently labelled S1 (labelled according to Chu and Cantor[15] and by a modified procedure, see Materials and Methods) on both "tight" and "loose" ribosomes. These two types of ribosomes are distinguished by the midpoints of the Mg^{2+} titration curves. The "tight" ribosomal couples are 50% associated at about 2.5 mM Mg^{2+} whereas "loose" couples require about 4-5 mM Mg^{2+} for 50% association.[16]

MATERIALS AND METHODS

A. Preparation of Ribosomes and Components.

Ribosomes were prepared from *E. coli* MRE600 cells as described previously.[17] Subunits free of S1 were prepared on 10-30% linear sucrose gradients according to Sobura et al.[2] After the final wash the subunits were resuspended in buffer containing 20 mM Tris, pH 7.8, 0.5 M NH_4Cl, 10 mM magnesium acetate and 1 mM dithiothreitol, heat activated at 37° for 50 min and cooled to 4°C over a period of 45 min to 1 hr. The 30S subunits were stored at 0° and used within 48 hr of preparation. An absorbance of 0.145 at 260 nm for a 0.001% solution was used to determine the ribosome concentration.

The 70S ribosomes, containing about 0.3 copy of S1 per ribosome, were either freed of S1 by poly C cellulose chromatography (PC ribosomes) or by dialysis (LS ribosomes) against low salt buffer (1 mM Tris, pH 7.4 for 18 hr[18]). The conditions for poly C cellulose treatment of ribosomes were the same as those

described for poly U treatment by Subramanian et al.[19] For some experiments the binding of S1 to the 70S particles was measured without removing residual S1. In the latter case, the total S1 concentration was calculated from the S1 added and the S1 present on the ribosomes. The S1 present on the ribosomes was determined from the equivalence point in the S1-ribosome titration.

Ribosomal protein S1 was prepared by poly C cellulose chromatography according to Sobura et al.[2]. The purified protein was stored at -80° in buffer containing 20 mM Tris pH 7.8 (at 20°), 10 mM Mg acetate, 1 mM EDTA and 0.1 mM dithiothreitol.

B. Fluorescence Labelling of S1.

Purified ribosomal protein S1 was labelled with 1,5 IAEDANS (iodoacetylethylenediamine (1,5)-naphthol sulfonate) according to the procedure of Chu and Cantor[15] (hereafter designated S1-CC) or by a modified procedure (S1-Mod). Chu and Cantor[15] dialyzed the labelled S1 against 6 M urea overnight and then against buffer to remove the urea. In the modified procedure the protein (1-2 mL) was dialyzed 3 hr against 6 M urea (T = 4°) and then passed over a Sephadex G-25 column (0.5 X 35 cm) equilibrated with 10 mM Tris buffer, pH 7.6, 20 mM $Mg(OAc)_2$, 250 mM NH_4Cl and 6 mM 2-mercaptoethanol.

C. SDS Disc Gel Electrophoresis.

Electrophoresis of S1 in acrylamide gels containing SDS was performed according to Weber and Osborn.[20] The gels were run (150 volts, 4°C) for about 1 hr until the tracking dye (0.001% Bromphenol Blue) was within 1 cm of the bottom of the tube. Gels were stained with Coomassie blue (1.25 g in 454 mL 50% methanol and 46 mL glacial acetic acid).

D. Poly U-Dependent Protein Synthesis Assay.

Poly U-dependent poly(Phe) synthesis in the presence of IF3 was performed as described by Sobura et al.[2]

E. Anisotropy Measurements.

Fluorescence anisotropy was measured using a photoelastic modulator (Morvue Electronics Systems, Tigard, OR) similar to the apparatus previously described.[21] The sample was placed in a 1 cm quartz cuvette. The sample fluorescence was excited with the 325 nm line of a He-Cd laser (325 nm, Model 4110 HUV,

Liconix, Mountain View, CA). Emission fluorescence was detected by a photomultiplier (9601 B, EMI, Plainview, NY) 90° to the exciting beam. The laser light passed through an interference filter (325 nm) before entering the cuvette. The emission was filtered through a sodium nitrite liquid filter to eliminate scattered light and a 520 nm long wavelength pass filter (λ = 520 nm, Ditric Optics, Inc., Hudson, MA) to eliminate any back ground fluorescence. A depolarizer preceded the photomultiplier. A programmable shutter was used to avoid photodegradation of the sample. The anisotropy of the sample was determined by taking the ratio of fluorescence intensities with the modulator on and off, after subtracting from both signals the voltage reading when the laser beam was blocked, ($\gamma = I_{on}/I_{off}$, usually <1). From equation 9 of Wampler and DeSa,[22] the anisotropy, r, is given by

$$r = \frac{2(1 - \gamma)}{1.104 + \gamma}$$

Fluorescence anisotropy is related to fluorescence polarization, P, by:

$$P = \frac{3r}{2 + r}$$

The entire fluorescence anisotropy apparatus was controlled by an Apple II microcomputer. The computer turned the modulator on and off, closed the shutter for dark voltage readings and for protection of the sample from photo damage, then triggered the Nicolet 2090-III-206 12-bit digital oscilloscope (Nicolet, Madison, WI) used for data acquisition. The computer processed the data from the Nicolet memory to obtain anisotropies. Typically, three runs of thirty data points (10 anisotropy values) each were acquired and processed to obtain a single averaged anisotropy value for each solution.

F. S1 Binding to 70S Ribosomes or 30S Subunits.

The equilibrium binding of S1 was measured as follows. A known concentration of fluorescently labelled S1 (0.106 μM) in a buffer containing 20 mM Tris, pH 7.6, 30 mM KCl, and $Mg(OAc)_2$ as indicated was titrated with increasing amounts of 70S ribosomes or 30S ribosomal subunits. All experiments were performed at 20°. The anisotropy was measured for S1 alone and after each addition of ribosomes. In one set of experiments, the 70S ribo-

somes were formed by mixing the isolated 30S and 50S subunits.

G. Data Fitting.

The equilibrium constant for the binding of IAEDANS-labelled S1 to 70S ribosomes and 30S subunits was determined from the anisotropy titrations. Since the fluorescence intensity was unchanged when ribosomes were added to the S1 solution, the anisotropy at any point in the titration can be expressed as:

$$r_{obs} = \frac{r_{S1}[S1] + r_{Rb-S1}[Rb-S1]}{[S1]_{Total}}$$

where r_{S1} is the anisotropy of free S1, determined before addition of ribosomes, and r_{Rb-S1} is the anisotropy of bound S1, determined at saturating ribosome concentrations. Since total S1 ($[S1]_T$) and total ribosome concentrations ($[Rb]_T$) are known, and $[Rb-S1] = [S1]_T - [S1]$, then

$$[S1] = [S1]_T (r_{obs} - r_{Rb-S1})/(r_{S1} - r_{Rb-S1})$$

By conservation,

$$[Rb-S1] = [S1]_T - [S1]$$

and

$$[Rb] = [Rb]_T - [Rb-S1].$$

In principle, therefore, the equilibrium constant

$$K_2 = \frac{[Rb-S1]}{[Rb][S1]}$$

can be determined from any point on the anisotropy titration curve once r_{S1} and r_{Rb-S1} are known. The r_{obs} vs. $[Rb]_T$ data were fit, however, with a Fletcher-Powell sum of squares minimization[23] using only one variable (K_2) for the entire titration curve. The quantity $(r_{obs} - r_{calc})^2$ was minimized in the fitting procedure, where

$$r_{calc} = \frac{-b + \sqrt{b^2-4ac}}{2a}$$

where

$$b = [Rb]_T \cdot K_2 \cdot D - [S1]_T \cdot D \cdot K_2 + D \text{ and } D = r_{S1} - r_{Rb-S1}$$

$$a = [S1]_T \cdot K_2$$

and

$$c = -r_{Rb-S1}^2 \cdot K_2 \cdot [S1]_T + r_{Rb-S1} \cdot K_2 \cdot D([S1]_T - [Rb]_T) - D(D + r_{Rb-S1})$$

H. Determination of Free Dye and Extent of Labelling.

The S1 prepared by the modified procedure was hydrolyzed[24] and analyzed by thin-layer chromatography on polyamide sheets. The solvent used was water:formic acid (100:3 vol/vol). Since the IAEDANS reaction was quenched with a large excess of 2-mercaptoethanol, any dye not bound to the Cys residue of the protein should be bound to the mercaptoethanol. The mercaptoethanol conjugated AEDANS and Cys conjugated AEDANS migrate quite differently in this solvent system.

The extent of labelling of the S1 was determined from absorbance measurements. A molar extinction coefficient of 6.1 X 10^3 M^{-1} cm^{-1} at 336 nm was used for AEDANS[25] and a value of $A_{280}^{1\%}$ = 7.67 was used for S1.[8]

RESULTS

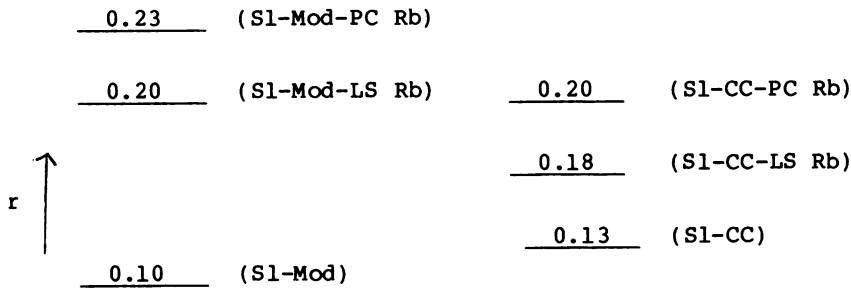
The initial anisotropy for AEDANS-S1 varied with the S1 preparation. When S1 was prepared by the modified procedure without extensive dialysis, the anisotropy ranged from 0.085 to 0.100 $\pm$ 0.005 for 10 preparations. The anisotropy for S1 prepared according to Chu and Cantor[15] was 0.12-0.13 $\pm$ 0.01. The thin-layer chromatography of S1 prepared by the modified procedure showed no detectable AEDANS that was not conjugated to the Cys residue. Standard solutions showed that amounts as small as 5% of the total AEDANS applied could be detected. Unconjugated dye could not account for the lower initial anisotropy of S1-Mod.

The dye/protein ratio determined from absorbance measurements was 1.1 $\pm$ 0.1 dye/S1. This is in agreement with Chu and Cantor.[15] The probable site of the reaction of the AEDANS is the more reactive SH group at position 349 of the S1 polypeptide chain.[8]

Sodium dodecyl sulfate (SDS) disc gel electrophoresis was run on the two labelled S1 samples, S1-CC and S1-Mod, as well as unlabelled S1. The S1 prepared according to Chu and Cantor[15] (S1-CC) did not co-migrate with unlabelled S1, but appeared to have a molecular weight about 10,000 daltons smaller than unlabelled S1. Such behavior had been observed previously (26 and A.J. Wahba unpublished results).

The activity of AEDANS labelled S1 was measured by Poly U-dependent poly(phe) synthesis in the presence of IF3. S1 is known to modulate this reaction by showing a progressive stimulation and inhibition of protein synthesis.[2] The activity of AEDANS-S1 was within 90% of that of unmodified S1.

The S1-ribosome complex also showed some variation in the anisotropy. For S1-CC and LS ribosomes the final anisotropy was 0.18 ± 0.005; for S1-CC and PC ribosomes the final anisotropy was 0.200 ± 0.005. S1-Mod and LS ribosomes gave a final anisotropy of 0.200 ± 0.005. The S1-Mod with PC ribosomes had a final anisotropy of 0.23 ± 0.01. These results are summarized in the diagram below:



To determine the state of the ribosomes, Mg^{2+} titrations were performed and the asssociation of the ribosomes was measured by light scattering.[21,27,28] The low salt dialyzed ribosomes were mostly "loose" couples with a midpoint in the titration curve of about 6 mM Mg^{2+}. About 20% of the ribosomes treated in this manner would not associate to 70S particles. The ribosomes treated by poly C cellulose chromatography were less than 15% "loose" couples. The midpoint in the titration curve was 2.8 mM Mg^{2+}. All ribosomes were heat-activated and the "loose" couples were active in protein synthesis.

The values for the equilibrium constants for S1-Mod binding to LS and PC ribosomes as a function of Mg^{2+} concentration are given in Table 1. Because of the small range and the variability of the anisotropy values for S1-CC binding to PC and LS ribosomes, the equilibrium constants were not calculated for these materials. Values are shown for ribosomes that were freed of S1 by poly C cellulose chromatography (PC ribosomes) and ribosomes

TABLE 1: Equilibrium Constants for S1-Mod Binding to 70S Ribosomes and 30S Subunits.*

Ribosomes	70S		30S
$[Mg^{2+}]$	PC "Tight"	LS "Loose"	From PC "Tight"
	K_{eq} (μM^{-1})		
2 mM			6.5 ± 0.5
5 mM	4.1 ± 0.9	150 ± 20	
6 mM	14 ± 2		
8 mM	72 ± 2		
10 mM	184 ± 3	140 ± 30	7.0 ± 0.6

* The errors given are calculated from the variance of the Fletcher-Powell curve fitting. At least four curves were used to obtain each equilibrium constant value.

freed of S1 by low salt treatment (LS ribosomes). For S1-Mod binding to 70S PC ribosomes there is a greater than 40-fold change in equilibrium constant for a doubling of the (Mg^{2+}) from 5 to 10 mM. The isolated 30S subunit and LS ribosomes show a much smaller effect of (Mg^{2+}) on the binding constant. When the isolated 30S subunits were recombined with 50S subunits, the Mg^{2+} dependence of S1 binding was restored and the binding was within experimental error of that for PC ribosomes. Figure 1 shows a comparison of the titration curves for PC ribosomes and 30S subunits as a function of Mg^{2+}.

DISCUSSION

Fluorescence anisotropy proves to be a useful technique for rapid determinations of equilibrium constants at very low reactant concentrations. This has allowed us to measure S1 interactions with ribosomes under conditions of varying Mg^{2+} concentration. Chu et al.[14] recently concluded that there was little change in fluorescence anisotropy when S1 bound to ribosomes and that the S1 had considerable rotational freedom. We confirm their results for similarily prepared materials, LS ribosomes and S1-CC. We find, however, that for PC ribosomes and intact S1 there is a substantial anisotropy change. The anisotropy of the 30S-S1 complex (0.23) approaches the limiting values for this dye measured in sucrose (60% w/v, 20°C) (0.24). This sug-

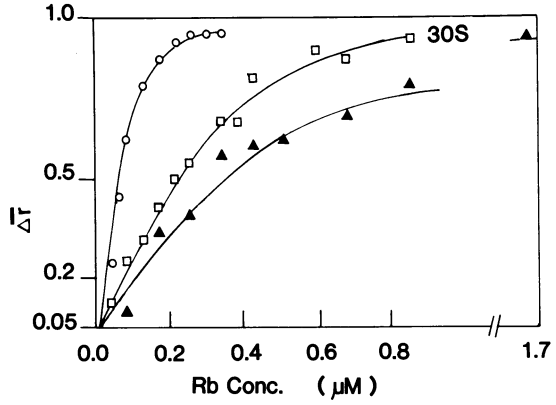


Figure 1: Fluorescence Anisotropy Change for the Reaction of AEDANS-S1 with Ribosomes. The open circles and solid triangles show the reaction of PC ribosomes with S1-Mod at 10 and 5 mM Mg^{2+}, respectively. The squares show the reaction of isolated 30S subunits with S1-Mod at 10 mm Mg^{2+}. The left axis is the normalized fluorescence anisotropy change. The abscissa is total ribosome concentration. T = 20°.

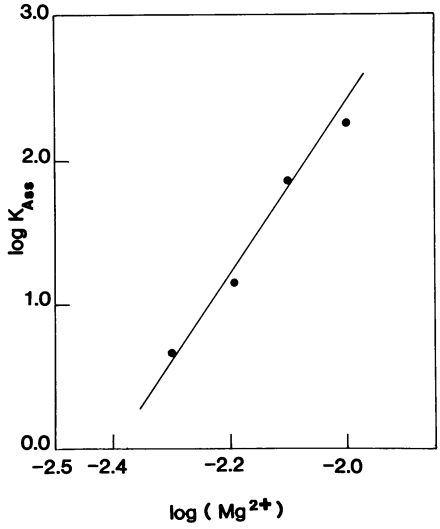


Figure 2: The Mg^{2+} Dependence for S1-Mod Binding to PC Ribosomes at 20°C. $K_{Ass.}$ is in units of μM^{-1}; (Mg^{2+}) is in units of M. The least-squares line has a slope of 6.0.

gests very limited rotational freedom for the S1 bound to the ribosome. The prolonged dialysis of the ribosomes at low salt used by Chu and Cantor[15] may have led to a conformational change in the ribosomes that is not completely reversible upon heat activation. We find a significant difference in the anisotropy change and the (Mg^{2+}) dependence of S1 binding for PC and LS ribosomes. The equilibrium constant for S1 binding to 70S ribosomal tight couples varied >40-fold from 5-10 mM Mg^{2+} and shows a 6^{th} power dependence on (Mg^{2+}). See Figure 2. This strong Mg^{2+} dependence reflects the difference in Mg^{2+} binding between ribosomes with and without S1 bound. The 6^{th} power dependence suggests that 6 more Mg^{2+} ions are bound per ribosomes when S1 is attached to the ribosome than when it is not. What is the source of this (Mg^{2+}) dependence? S1 has been shown to be an elongated protein that can be modelled by a prolate ellipsoid with axial ratio of 10 to 1.[1] It has also been suggested that S1 is a flexible protein with at least two independent domains.[15,19,29] It seems likely that S1 could undergo a large conformational change. However, a titration of labelled S1 with Mg^{2+} shows only a small increase (5%) in anisotropy from 2-10 mM Mg^{2+}. Chu and Cantor[15] found that the rotational correlation time for IAEDANS labelled S1 did not change upon addition of Mg^{2+}. It seems unlikely that very large conformational changes are occurring, at least near the IAEDANS binding site. The ribosome-binding domain of S1 does not, however, contain the reactive SH group.[8,30,31] S1 has several clusters of negatively charged amino acids[8] which could bind Mg^{2+}. It has also been reported[32] that S1-mRNA complexes aggregate in the presence of Mg^{2+}. Further studies are in progress to determine if S1 is in fact a Mg^{2+} binding protein. Preliminary studies in our laboratory, however, show no strong Tb^{3+} binding by S1. The 30S subunits and ribosomal loose couples did not show such a pronounced Mg^{2+} effect. However, 70S particles reconsitituted from isolated 30S and 50S subunits retained the (Mg^{2+}) dependence. The observed Mg^{2+}dependence was somewhat less than that observed for 70S ribosomes described above, however, only about 70% of the 30S subunits formed 70S particles when mixed with the corresponding 50S particles. When the concentration of

30S particles still present is taken into account, the (Mg^{2+}) dependence is nearly the same as that for native 70S particles. This suggests that the critical Mg^{2+} binding sites are located on the intact 70S ribosome. The change in Mg^{2+} binding may not be due to specific sites so much as a change in the total concentration of bound Mg^{2+} ions. Such changes may be due to conformational changes in the ribosome, in particular in the rRNA. Presumably the 50S subunit distorts the 30S subunit or there is a direct interaction of the S1 with both the 30S and 50S subunits, probably at an interface. While there is indirect evidence that S1 binds at the 30S-50S interface, Boileau et al.[10] did not find S1 cross-linked to any 50S proteins in the 70S ribosome. They did find, however, that cross-linking to the 70S ribosome, as compared to the isolated 30S subunit, resulted in an increased yield of cross-linked proteins S1 and S10. This suggests an alteration in arrangement of proteins in the 70S ribosome or a change in reactivity.

It has been suggested that binding of S1 itself can produce a conformational change in the isolated 30S subunit.[1,33,34] Ultracentrifuge studies[33,35] have shown a slower sedimentation for S1-containing 30S particles relative to those lacking S1. Whether this is due to a conformational change in the 30S particle or to frictional drag from the elongated protein itself is not clear. Michalski et al.[36] have shown that binding of S1 to 30S subunits alters the reactivity of five ribosomal proteins to enzymatic iodination. Proteins S7, S12, and S21 were observed to be more susceptible to enzymatic iodination after S1 was bound whereas proteins S3 and S19 were less susceptible. Our results show that the S1 binding to the isolated 30S subunit does not depend on Mg^{2+}.

Laughrea and Moore[37] and Draper and von Hippel[13] have reported binding constants of 30S and 70S particles for S1 protein. They found no significant differences between 30S subunits and 70S ribosomes at 5 mM Mg^{2+}. We also conclude that there is only a small difference in binding at this (Mg^{2+}); however, the (Mg^{2+}) dependence of the binding is very different for the 30S subunits and the 70S ribosomes and the two binding curves happen to cross near 5 mM Mg^{2+}. Draper and von Hippel[13]

reported the binding constant to be about 2 orders of magnitude higher than that reported by Laughrea and Moore.[37] Both studies determined the equilibrium constants by sedimentation velocity. Draper and von Hippel[13] assumed that equilibration is fast relative to the rate of sedimentation and obtained a K_{eq} value at 5 mM Mg^{2+} (6° and 18°C) of about 200 μM^{-1}, larger than that reported here. Laughrea and Moore[37] assumed that sedimentation was slow compared to the rate of attainment of equilibrium and the binding constant they obtained, about 2 μM^{-1} at 5 mM Mg^{2+} and 0°C, is smaller than our value. Both of these studies used higher concentrations of monovalent cations than used in this study. The results we report do not require such equilibration rate assumptions, since we found the anisotropy to be constant within 30 sec after each addition of ribosomes.

At high (Mg^{2+}) $\Delta G°$ = -11 kcal/mol for the binding of S1 to 70S ribosomes and the binding is stabilized ($\Delta G°$) by about -2 kcal/mol with respect to binding to 30S subunits. Whether the source of this stabilization is due to protein-rRNA interactions or protein-protein interactions is unclear. Yuan et al.[38] found that the colicin fragment of 16S rRNA bound S1 with a $\Delta G°$ = -9 kcal/mol. They also observed, however, that the binding was stronger at lower Mg^{2+}. For the intact ribosome, we find stronger binding at higher Mg^{2+}. Lipecky et al.[39] found that the binding of polynucleotides to S1 was Mg^{2+} dependent at low ionic strength (< 90 mM) and increased with (Mg^{2+}), reaching a maximum at 10 mM Mg^{2+}. Laughrea and Moore[37] reported little difference in binding affinity between intact 30S ribosomes and 30S subunits from which the colicin rRNA fragment had been removed. They attributed most of the free energy to protein-protein interactions, but removal of the 49 nucleotide colicin fragment may alter the conformation of the 30S subunit. Our results on the difference between 30S and "tight" and "loose" 70S ribosomes and the (Mg^{2+}) dependencies suggest that conformational changes in the ribosome play a very important role in S1 binding.

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